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Title: Confirmation of Phenylbutazone in Bovine Kidney by LC/ESI-MS/MS		
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Contents

A. INTRODUCTION

1. Theory

Phenylbutazone (PBZ) is extracted from kidney tissues into aqueous ammonium hydroxide. The aqueous extract is partitioned with mixed ethers to remove lipids, then acidified, and PBZ are extracted into a tetrahydrofuran-hexane (THF-hexane) solution. This extract is further purified by silica gel solid phase extraction (SPE) chromatography. The eluate is evaporated, then redissolved in mobile phase of high performance liquid chromatography (HPLC) and analyzed by HPLC/Electrospray ionization MS/MS (HPLC-ESI-MS/MS) in negative ion selected reaction monitoring (SRM) mode. Confirmation is based on comparison of HPLC retention times and MS/MS daughter ion ratios against those determined for a reference standard.

2. Applicability

This method will confirm phenylbutazone in bovine kidney at $\geq$ 50 ppb.

B. EQUIPMENT

Note: Equivalent equipment may be substituted for the following.

1. Apparatus

a. Centrifuge - Super-speed Refrigerated Centrifuge with Rotor #876 capable of attaining 5000 rpm in 10 min., (Refrigeration is not required), Cat. No. 20671-007, VWR Scientific, San Francisco, CA.

b. Eppendorf pipettors - Variable volume pipettes: Fisher Scientific: 2 - 20 µL (Cat. No. 05-402-46), 10 -100 µL (Cat. No. 05-402-48), 50 - 200 µL (Cat. No. 05-402- 49), 100 -1000 µL (Cat. # 05-402-50) and 500 - 2500 µL (Cat. No. 05-402-51).

c. SPE columns - Silica solid phase extraction columns (6 cc, 500 mg), Part No. 43400, Waters Corp.

d. Nitrogen Evaporator - Organomation Associates, Berlin, MA.

e. Vortex mixer - Fisher Scientific.

f. Balance - PM 300, Mettler.

g. PVDF membrane filter - 0.45 µm, Cat. No. 254-643, Labsource, Chicago, IL.

h. Syringe filter - 0.2 µm PVDF membrane syringe filter, Part No. 4450T, Gelman, Ann Arbor, MI.

i. Glassware - Volumetric Glassware includes 10 mL, 25mL, 50 mL, 100 mL, and 250 mL graduated cylinders.

j. Test tubes - 16 mm x 125 mm (20 mL), 16 mm x 100 mm (15 mL), and 20 mm x 150 mm (36 mL) disposable Borosilicate glass culture tubes, Kimble.

k. Test tubes - 50 mL polyallomer tube with polypropylene screw closure, Cat. No. 3139-0050, Nalge Company, Rochester, NY 14602-0365.

l. SPE vacuum manifolds - Supelco separation technology.

m. Shaker - Eberbach Corporation, Ann Arbor, Michigan.

2. Instrumentation

a. Mass spectrometer - Thermo Finnigan, TSQ Quantum.

b. HPLC equipped with a quaternary pump and auto-injector - Thermo Finnigan Surveyor MS pump and Thermo Finnigan Surveyor Auto Sampler.

c. Analytical column - YMC ODS-AQ, 120 Å, 2 x 100 mm, 3 µm, Part No. AQ 12S031002 WT, Waters Corp., Milford, MA.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents/solutions may be substituted for the following.

1. Reagents

a. Ethyl ether - Reagent grade, Cat. No. 107-1, Burdick & Jackson.

b. Petroleum ether - Reagent grade, Cat. No. 317-4, Burdick & Jackson.

c. Tetrahydrofuran (THF) - Reagent grade, Cat. No. 340-2, Burdick & Jackson.

d. Hexane - Reagent grade, Cat. No. H303-4, Fisher Scientific.

e. Acetonitrile (ACN) - HPLC grade, Cat. No. AH015-4, Burdick & Jackson.

f. Water - Deionized water, HPLC Grade, Millipore Rx system.

g. Methanol (MeOH) - HPLC grade, Cat. No. AH230-4, Burdick & Jackson.

h. Ammonium Acetate (NH_4OAc) - HPLC grade, Cat. No. A639-500, Fisher Scientific.

i. Ammonium Hydroxide (NH_4OH) - 30% Cat. No. 9721-01, J. T. Baker.

j. Acetic acid - HPLC grade, Cat. No. AX0074.6, E. M. Science.

k. Hydrochloric acid (HCl) - Reagent grade, Cat. No. A144-212, Fisher Scientific.

2. Solutions

a. 7.5% Ammonium Hydroxide Solution:

Dilute 25 mL of 30% ammonium hydroxide to 100 mL with deionized water

using a graduated cylinder.

b. 0.01 M ammonium acetate in 0.5% acetic acid:

Add 0.77 g ammonium acetate to a 1L volumetric flask; add enough DI water to dissolve ammonium acetate. Then add 5 mL acetic acid and dilute to volume with DI water (0.5% acetic acid, v/v). Filter through a 0.45 µm PVDF membrane filter prior to use.

c. LC Mobile Phase:

Combine equal volumes of acetonitrile and 0.01M ammonium acetate in 0.5 % acetic acid (C.2.b.) and mix. Alternatively, use the HPLC solvent delivery system to deliver the above 50:50 mixture.

d. THF/Hexane (1:4):

Add 60 mL of THF to 240 mL of hexane

e. Solvent 1 - acetonitrile:0.2N acetic acid (50:50):

Dilute 3 g or 2.9 mL of conc. acetic acid (17.4N) to 250 mL with deionized water. Add this solution to 250 mL acetonitrile. Mix well.

f. 6N HCl:

Dilute 12N HCl 1 to 1 with deionized water.

D. STANDARDS

Note: Equivalent standard/solutions may be substituted for the following.

1. Source

Phenylbutazone ($C_{13}H_{20}N_2O_2$, MW 308 and CAS 50-33-9) standard, Cat. No. 53567, ICN Biomedical Inc., Aurora, OH.

2. Preparation of Standards

Note: If purity is less than 100%, make corrections based on the actual purity provided.

a. Stock PBZ Standard Solution (500 µg/mL):

Accurately weigh 50.0 ± 0.1 mg PBZ standard into a 100 mL volumetric flask. Dissolve and bring to volume with methanol. Stable for two months at 2 - 8 ºC.

b. Working Standards (5 µg/mL):

For 5 µg/mL, dilute 50 µL of 500 µg/mL stock standard (D.2.a.) solution to 5 mL with methanol in a volumetric flask.

Note: This solution is stable for two months if stored at 2 - 8 °C.

c. LC/MS External Standards (50 ng/mL):

i. 500 ng/mL:

Dilute 5 µL of 500 µg/mL stock standard solution (D.2.a.) to 5 mL with solvent 1 (C.2.e.) in a volumetric flask.

ii. 50 ng/mL:

Dilute 100 µL of 500 ng/mL standard solution (D.2.c.i) with 900 µL of solvent 1 (C.2.e.) in a LC sample vial.

Note: These solutions must be prepared daily.

E. SAMPLE PREPARATION

After removing excessive fat from kidney sample, cut it into smaller pieces and homogenize to fine paste with a mechanical food processor. Transfer homogenized sample into plastic bags and store in the freezer at -20 ºC. Let the sample partially thaw prior to analysis.

F. ANALYTICAL PROCEDURE

1. Samples extraction and cleanup procedure

a. Weigh 5.0 ± 0.1 g of partially thawed homogenized kidney sample into a 50 mL polypropylene centrifuge tube.

Note: Prepare positive and negative controls as follows:

i. Weigh 5.0 ± 0.1 g blank tissues into two 50 mL polypropylene centrifuge tubes.

ii. Prepare the 50 ppb positive control by fortifying one of the tubes with 50 µL of 5 µg/mL PBZ working standard (D.2.b.). Vortex vigorously for 10 sec.

b. Add 5 mL deionized water and 0.5 mL ammonium hydroxide solution (C.2.a.) to tube.

c. Vortex vigorously for 30 sec.

d. Add approximately 10 mL ethyl ether to tube, cap, and vortex for 10 sec.

e. Add 10 mL petroleum ether to tube, cap and shake vigorously for 30 sec.

f. Centrifuge tube for 10 min at 5000 rpm at room temperature.

g. Remove and discard top ether layer using a Pasteur pipette.

h. Vortex tissue and aqueous layer to homogenize.

i. Add 1 mL 6N HCl (C.2.f.) to each tube, cap and vortex for 30 sec to ensure

that the acid was incorporated throughout the tissue mixture.

j. Add 20 mL THF/hexane (C.2.d.) to tube, cap and mix vigorously on a shaker for 10 min. (Set shaker to Hi)

k. Centrifuge for 10 min at 5000 rpm.

l. Prepare silica SPE cartridge by passing 3 mL of THF/hexane (C.2.d.)
Note: Do not let the column go dry.

m. Pass the upper organic layer from sample tube through the prepared SPE cartridge and collect the eluate in a 50 mL beaker. (Discard contents from the centrifuge tube).

n. Rinse the cartridge with 3 mL THF/hexane (C.2.d.) and collect the eluate in the same beaker as in step n.

o. Transfer the eluate from the beaker to a 20 x 150 mm (36 mL) glass test tube.

p. Evaporate the combined eluate to dryness with a gentle stream of nitrogen in an approximately 50 °C water bath.

q. Dissolve residue in 1 mL solvent 1 (C.2.e.) by vortexing for 1 min.

r. Filter through a 0.2 µm Acrodisc LC 13 PVDF syringe filter or equivalent into a LC vial.

Note: For a 50 ppb recovery, the concentration of phenylbutazone in the above filtrate will be 250 ng/mL.

s. The sample is ready for HPLC-ESI-MS/MS analysis.

Note 1: If necessary, the sample extract may be diluted to better approximate the concentration of external standard.

Note 2: Stopping point: If the sample extract is not analyzed on the same day, store in a -20 ºC freezer and analyze the following day.

2. Instrumental Parameters

Note: The instrument parameters listed here are typical values for the instrument specified. The analyst should optimize parameters for the specific instrument being used.

a. HPLC conditions:

Column temperature	25 °C
Mobile Phase	See Section C.2.b.
Flow rate	0.25 mL/min
Injection volume	10 µL
Run Time	20 min

b. Mass Spectrometer Parameters:

Mode of Operation	Negative Ion Mode
EM Voltage	-1279 V
Collision cell pressure	1.0 mTorr
Tube lens-offset	-118 V
Auxiliary gas pressure	0 bar
Sheath gas pressure	35 bar
Capillary temp	251 °C
*Collision energy	20 V
Spray voltage	3197.4 V

*Note: Collision energy should be adjusted so that m/z 307 ion has largest abundance.

c. Data Acquisition Selected Monitoring (SRM) mode—Precursor ion 307.

Ion 131, scan width 0.6d, scan time 0.15 sec.

Ion 160, scan width 0.6d, scan time 0.15 sec.

Ion 188, scan width 0.6d, scan time 0.15 sec.

Ion 279, scan width 0.6d, scan time 0.15 sec.

Ion 307, scan width 0.6d, scan time 0.15 sec.

3. MS Optimization
    a. Verify that the instrument is in an acceptable state of tune before attempting confirmatory analyses.
    b. Perform a full MS scan by using syringe injection analysis of phenylbutazone stock solution (5 µL/min fused with 250 µL/min mobile phase) and obtain the centroid of the precursor ion (m/z 307). Perform a MS/MS scan using the precursor ion to obtain the product ions (m/z 131, 160, 188 and 279).
    c. Inject 10 µL of 50 ng/mL PBZ standard into the HPLC and analyze. Sufficient sensitivity should be present (>20x background signal) for the m/z 307 ion along with the product ions of m/z 279, 188, 160 and 131.

4. Confirmation Analysis Injection Sequence
    a. For sample analysis use the following injection sequence:
        i. External standard
        ii. Solvent blank
        iii. Negative tissue control (blank tissue)
        iv. Positive tissue control (recovery)
        v. Sample(s)
        vi. Solvent blank
        vii. External standard

        Note: Normally carry-over is observed when the solvent blank is injected after a high concentration of phenylbutazone is analyzed. It is highly recommended to inject a solvent blank after each unknown sample is analyzed.

5. Confirmation
    a. Plot and integrate ion chromatograms for all standards, controls, and samples injected. Record analyte retention time and ion abundances for all monitored ions. Calculate ion abundance ratios relative to m/z 307.
    b. Confirmation of any sample requires that the following criteria be met:
        i. The negative control extract does not show confirmable PBZ.
        ii. The retention time of the PBZ peak in the sample must be within ± 4% of that determined for the reference standard (fortified control).

        Note: If necessary, the external standard may be used as the reference standard instead of the fortified control.

iii. All four product ions and the precursor ion (131, 160, 188, 279 and 307) must be present in the sample and reference standard.

iv. All product ion ratios calculated for the sample must match those of the reference standard within a relative difference of ± 20% for ratios between 20 - 100%, and within ± 50% for ratios <20%.

G. CALCULATIONS (not applicable)

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, disposable gloves, lab coats.

2. Hazards

Reagents / Solutions	Hazard	Recommended Safe Procedure
Methanol, diethyl ether, petroleum ether, tetrahydrofuran.	Flammable and poisonous	Wear gloves, work in fume hood.
	Diethyl ether and tetrahydrofuran can form explosive peroxides after extended exposure to air.	Do not allow accumulations of diethyl ether or tetrahydrofuran dry out.
HPLC Mobile phase containing acetic acid, ammonium acetate, acetonitrile and water.	Irritation to skin, eyes, nose, mouth, throat and mucous membrane and may cause burns to skin.	Wear gloves, work in hood. Use protective eyewear.

3. Disposal Procedures

Reagents / solutions	Hazard	Recommended Safe Procedure
Methanol, diethyl ether, petroleum ether	Flammable and poisonous	Collect in a tightly sealed container and store in the flammable liquid storage area for disposal in accordance with local, state, and Federal regulations.
HPLC Mobile phase containing acetic acid, ammonium acetate and acetonitrile	Irritation to skin, eyes, skin, nose, mouth, throat and mucous membrane	Neutralize and transfer waste in a tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. No false positives from blank tissues.
   b. No false negatives from recoveries fortified at 50 ppb.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Reconstituted extract	Filter prior to the injection into HPLC-MS for analysis.

3. Readiness To Perform (FSIS Training Plan)
   a. Familiarization
      i. Phase I, Standard(s): Analyze in duplicate a 50 ppb phenylbutazone standard solution by using LC/ ESI-MS/MS, and obtain four product ions 131, 160, 188 and 279 and the precursor ion 307 of negative ions in SIM mode. Repeat this analysis on three different days to verify parameters.
      ii. Phase II, Analyst fortified sample extract(s): Conduct duplicate analyses of one tissue blank extract and one 50 ppb PBZ fortified tissue sample recovery on three separate days.

      Note: Phases I and II can be performed concurrently.

iii. Phase III, Check samples for analyst accreditation:
(a) A minimum of 8 blind check samples. At least one check sample should be blank. Samples should be spiked at 50 ppb level.

(b) Report analytical findings to Supervisor/Quality Assurance Manager (QAM).

(c) Notification from the QAM is required to commence official sample analysis.

4. Intralaboratory check samples

a. System, minimum contents.

i. Frequency: 1 per week as samples analyzed.

ii. Records are maintained.

b. Acceptability criteria: Refer to section I.1 above.

If unacceptable results are obtained, then:

i. Stop all sample analysis by the analyst.

ii. Take corrective action.

5. Sample set must include:

a. External standards.

b. 50 ppb positive control (recovery).

c. Tissue blank.

d. Sample extract(s).

6. Sensitivity

Minimum Proficiency Level (MPL): 50 ppb.

J. WORKSHEET

The following is an example worksheet.

PHENYLBUTAZONE CONFIRMATION WORKSHEET

Name of analyst		Sample No.	
Date Started		Form No.	
Date Completed		Tissue Code	
LC/MS ID		Dilution Factor	
Injection Volume		Positive/Negative	
Run Time			

Confirmation

Date of Analyses:

Date of Calculation:

Unknown Sample — File name:

Vial No.	RT(min)	m/z	Rel. ion %
		131	
		160	
		188	
		279	
		307	
		131	
		160	
		188	
		279	
		307	

___ ppb Fortified Tissue Sample Recovery — File name:

Vial No.	RT(min)	m/z	Rel. ion %	% Diff. Rel. ion	% Diff. RT
		131			
		160			
		188			
		279			
		307			
		131			
		160			
		188			
		279			
		307			

K. APPENDIX

1. Chromatograms

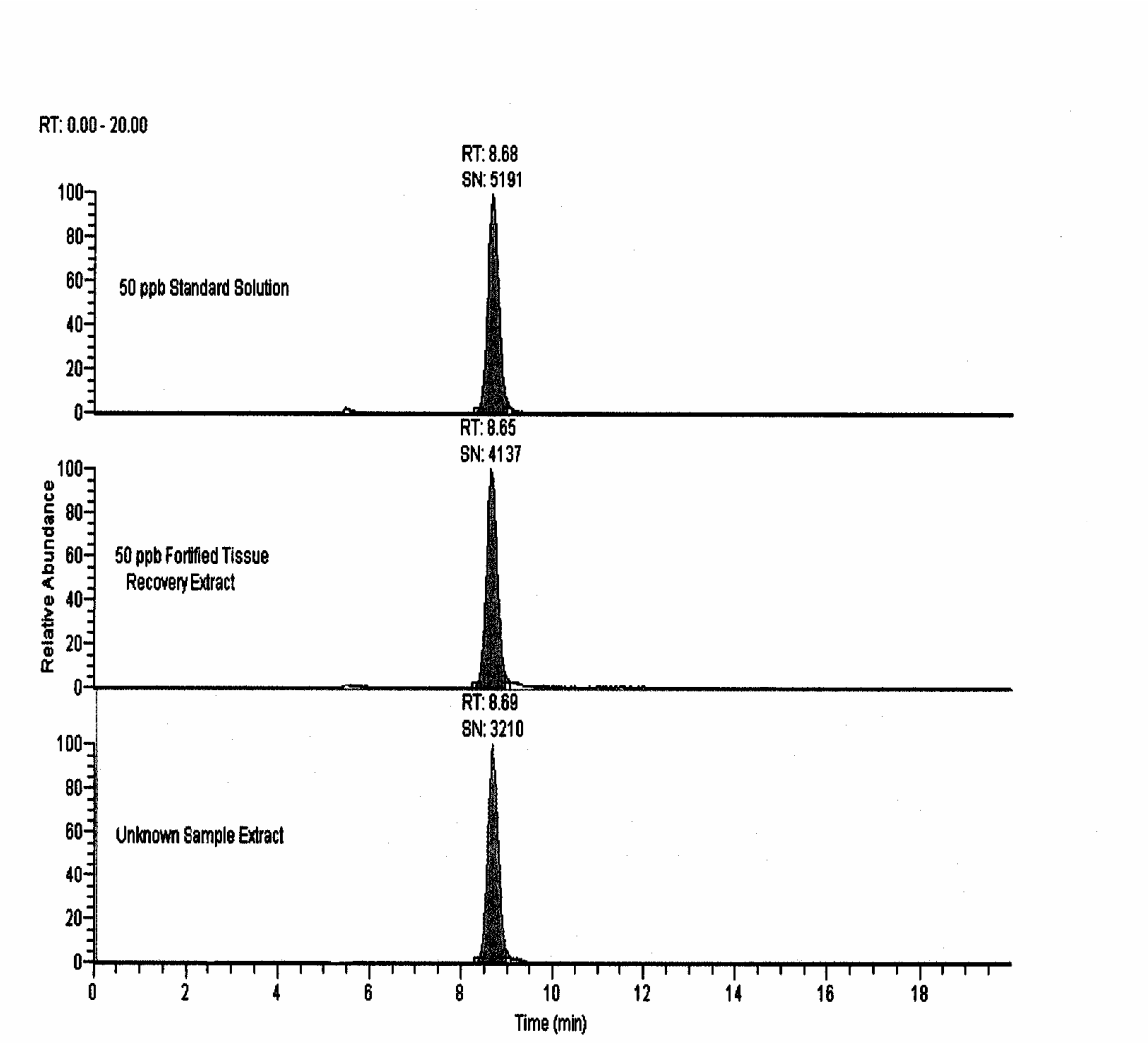
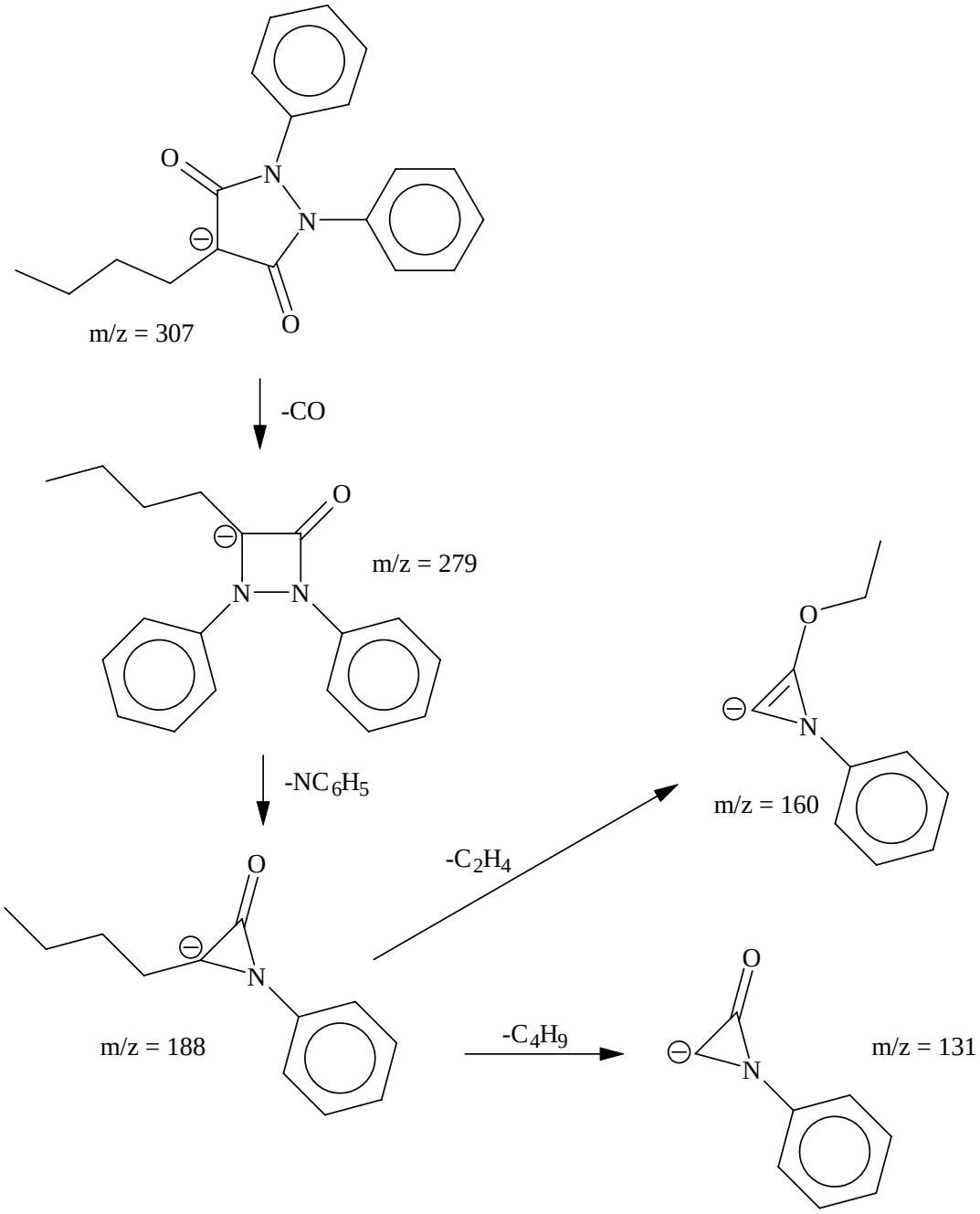


Figure 1**.** Chromatograms of 50 ppb phenylbutazone standard, 50 ppb fortified tissue recovery extract and unknown sample extract.

2. Proposed fragmentation pattern of phenylbutazone



3. Reference

Susan B. Clark, Sherri B. Turnipseed, Gene J. Nandrea, Mark R. Madson, Jeffrey A. Hurlbut and John N. Sofos. Confirmation of Phenylbutazone Residues in Bovine Kidney by Liquid Chromatography/Mass Spectrometry, J. AOAC Int. (2002) 85 (5), 1009 - 1014.

L. APPROVALS AND AUTHORITIES

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